Performance of commercially available gels for protein characterization by capillary gel electrophoresis with UV detection on the Agilent 7100 CE System

Application Note

Biopharm

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Abstract

In this Application Note, separation matrices for protein characterization by capillary gel electrophoresis (CGE) with UV detection from two suppliers, Beckman Coulter and Advanced Analytical, were compared on the Agilent 7100 CE system. Two different sample sets were analyzed: first, a protein size standard and BSA as a test protein for molecular weight determination; second, a reduced antibody standard for quantification of light chain, non-glycosylated heavy chain and heavy chain. To get an estimate for the intermediate precision, these experiments were performed with two different gel lots and capillary batches each. Across these experiments, the gels from both suppliers showed a similar performance. Impurity detection experiments were done with a low molecular weight protein spiked into a non-reduced antibody standard sample. With the gels of both suppliers it was possible to detect this protein down to a level of 0.1 %.

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Introduction

Capillary gel electrophoresis (CGE) is a widely used tool for the size-based analysis of protein. Due to several advantages with regard to automation, reproducibility and resolution, it has replaced the classical technique sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in many labs, especially in the biotechnology industry[1,2]. CGE it is now routinely used in the quality control environment to assess purity and integrity of therapeutic proteins including monoclonal antibody (mAb). Commercially available dextran-based separation matrices are used for these separations. An established product is the SDS Gel Buffer from Beckman Coulter[3,4]. Recently, new gels for CGE with UV detection were introduced onto the market by Advanced Analytical. These gels were originally developed for multi-capillary instruments[5].

This work is a detailed comparison of the performance of both separation matrices on the Agilent 7100 CE system. Experiments were performed with the same samples and methods as described in Nunnally *et al.*[4,6] to enable a direct reference to data obtained on instruments from other suppliers. Typical CGE applications were covered like (i) molecular weight determination with a protein molecular weight standard and BSA as a test protein, (ii) quantification of main antibody components under reducing conditions, (iii) low-level impurity detection in an intact (non-reduced) antibody sample.

Experimental

Materials

SDS-MW Size Standard, IgG Control Standard, 10 kDa Internal Standard, SDS Sample Buffer, SDS Gel Buffer, 0.1 M HCl and 0.1 N NaOH were from Beckman Coulter (Fullerton, CA, USA). Protigel A and P protein gels and protein capillary conditioning solution were from Advanced Analytical (Ames, IA, USA). BSA, β-lactoglobulin (bLG), iodoacetamide and 2-mercaptoethanol were from Sigma (Taufkirchen, Germany). All other materials and instrumentation were from Agilent Technologies (Waldbronn, Germany).

Sample preparation

The SDS-MW Size Standard, BSA and reduced IgG Control Standard were prepared as described in Nunnally *et al*[4]. Prior to CE analysis, 100 µL samples were transferred into 100 µL polypropylene sample vials.

Non-reduced IgG Control Standard samples spiked with bLG were prepared as follows:

1. Thaw a 47.5 µL aliquot of the IgG Control Standard at room temperature
2. Add 1 µL 10 kDa Internal Standard to the microvial
3. Add 1 µL of bLG dilution (in SDS Sample Buffer) to the microvial
4. Add 2.5 µL of 250 mM iodoacetamid (freshly prepared), cap tightly and mix thoroughly
5. Centrifuge at 300 × g for 1 min
6. Heat capped microvial to 70 °C for 10 min
7. Cool microvial in a room temperture water bath for 3 min
8. Transfer 50 µL of the prepared sample into a 100 µL polypropylene sample vial

Samples were prepared and used on the same day. One sequence consisted of two blank runs followed by six sample runs. Each individual sample vial was used for six injections only. Blanks were prepared in the same way as the samples but with SDS Sample Buffer added instead of SDS-MW Size Standard, BSA and IgG Control Standard, respectively.

CGE with Beckman Coulter gel

Fifty µm id bare fused silica capillaries with a total length of 33 cm and an effective length 24.5 cm were used for CGE runs with the SDS Gel Buffer from Beckman Coulter. Once a day, capillaries were conditioned as follows: high pressure flush at 2 bar with 0.1 N NaOH for 10 min, with 0.1 N HCl for 5 min and with water for 2 min; high pressure flush at 4 bar with SDS Gel Buffer for 10 min; water dip for both electrodes; voltage equilibration at 16.5 kV for 10 min with 5 min ramping.

Prior to every run, capillaries were conditioned as follows: high pressure flush at 4 bar with 0.1 N NaOH for 3 min, with 0.1 N HCl for 1 min, with water for 1 min and with SDS Gel Buffer for 10 min; water dip of both electrodes. Samples were injected electrokinetically by applying –5 kV for 20 sec and, after a water dip of the inlet electrode, separated by applying –16.5 kV (–500 V/cm) for 30 min (reduced samples) or 40 min (non-reduced samples).

Two bar pressure was applied to both inlet and outlet vials during the run. After use, capillaries were conditioned as follows: high pressure flush at 4 bar with 0.1 N NaOH for 15 min; high pressure flush at 3.5 bar with 0.1 N HCl for 5 min and with water for 10 min. All flushes were done in forward direction that is, pressure was applied to the inlet vial. The capillary temperature was kept at 25 °C.

The detection wavelength was 220 nm with a bandwidth of 20 nm (no reference wavelength) and a response time of 1 sec. For all reagents, 2 mL glass vials were used. The fill volume was 1.2 mL, except for the water dip vials that contained 1.6 mL water and the waste vials that contained 0.6 mL water. Three separate waste vials were used for the collection of 0.1 N NaOH, 0.1 N HCl/water and SDS Gel Buffer, respectively. SDS

Gel Buffer containing inlet and outlet home vials were exchanged after every sequence of eight runs.

CGE with gels from Advanced Analytical

Two different protein gels are available from Advanced Analytical: the Protigel P for the separation of proteins over a wide molecular weight range and the Protigel A for the separation of IgG non-glycosylated heavy chain and heavy chain[7]. According to these recommendations, CGE runs with molecular weight markers and BSA were done with the Protigel P and runs with the IgG standard with the Protigel A. The same analysis conditions were employed for both Protigel A and Protigel P, except for the pressure applied during the run (see below).

Bare fused silica capillaries with an id of 75 µm, a total length of 33 cm and an effective length of 24.5 cm were used. Before use and then every eight runs, capillaries were conditioned as follows: after adjusting the capillary temperature to 50 °C, high pressure flush at 3.5 bar with 1 N NaOH for 15 min; then, after readjusting the capillary temperature to 25 °C, high pressure flush at 3.5 bar with 0.1 N NaOH for 5 min, with protein capillary conditioning solution for 10 min and with Protigel for 10 min.

Prior to every run, capillaries were conditioned as follows: high pressure flush at 3.5 bar with Protigel for 5 min and then voltage equilibration at –10 kV for 5 min with 2.5 min ramping. Samples were injected electrokinetically by applying –5 kV for 20 sec and, after a water dip of the inlet electrode, separated by applying –10 kV (–303 V/cm) for 30 min. Two bar pressure was applied to both inlet and outlet home vials during the runs with Protigel P only. This was necessary to avoid frequent run failures due to current instabilities (data not shown).

After use, capillaries were conditioned as follows: high pressure flush at 3.5 bar with 0.1 N NaOH for 5 min and with water for 5 min. All flushes were done in forward direction (that is, pressure was applied to the inlet vial). The capillary temperature was kept at 25 °C, except for the conditioning step with 1 N NaOH. The detection wavelength was 200 nm with a bandwidth of 16 nm (no reference wavelength) and a response time of 1 sec. For all reagents, 2 mL glass vials were used. The fill volume was 1.5 mL, except for the water dip vials that contained 1.6 mL water and the waste vials that were empty. Four separate waste vials were used for the collection of 1 N NaOH, 0.1 N NaOH, Protein capillary conditioning solution and Protigel, respectively. Protigel containing inlet and outlet home vials were exchanged every eight runs.

Results and discussion

Molecular weight determination

The molecular weight of BSA was determined with a set of protein molecular weight markers using CGE gels from Beckman Coulter and Advanced Analytical, respectively. Each sample was measured six times and each set of analyses was done with two capillary batches and two gel lots to get an estimated for the intermediate precision (day-to-day precision).

Example electropherograms are shown in Figures 1 and 2 and results are summarized in Tables 1, 2 and 3. The peak pattern obtained with the gels from both suppliers was similar (Figures 1 and 2). In terms of relative migration time (RMT), results obtained with the gel from Beckman Coulter were in the

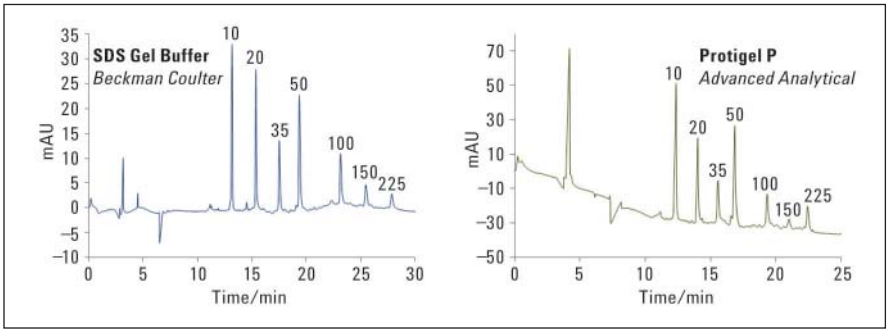

Figure 1
Representative electropherograms of the molecular weight standards. Molecular weights of protein markers are indicated (in kDa).

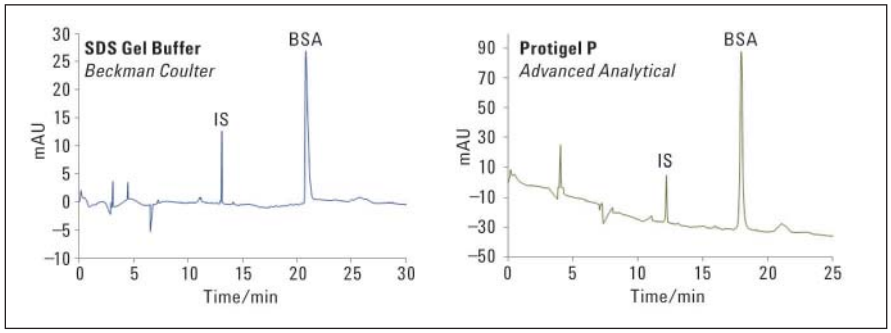

Figure 2
Representative electropherograms of BSA. IS, internal standard.

range of the data published by Nunnally *et al.*[4] for proteins ≤ 50 kDa and slightly increased for proteins > 50 kDa (Tables 1 and 3). These small deviations could be due to the different capillary effective length used in both cases (Nunnally *et al.*[4]: 20.2 cm; here: 24.5 cm). The RMT repeatability (intra-assay precision) for the gel from Beckman Coulter was between 0.03–0.19 RSD% and thus within the range of data reported by Nunnally *et al.*[4] (Tables 1 and 3).

RMT values obtained with the Protigel P from Advanced Analytical were different in comparison to the Beckman Coulter gel (Tables 1–3). This was not surprising given the different composition and viscosity of both gels, as well as the different experimental conditions used with regard to capillary diameter, preconditioning and separation voltage (cf. Experimental). Different RMT values in comparison to the gel from Beckman Coulter were also reported for a gel from a third supplier, the Optima Protein Run Buffer from Groton Biosystems[6]. The RMT repeatability with the Protigel P was between 0.01–0.11 RSD% and thus slightly better compared to the gel from Beckman Coulter (Tables 1–3).

For the molecular weight determination of BSA, plots of protein marker molecular weight against average RMT were prepared and the data were fitted to 3rd order polynomial trendlines (Figure 3). With the gels from both suppliers R-squared values ≥ 0.999 were obtained (Table 3). With this procedure a much better fit to the data was obtained than with the linear plot of log(MW) against 1/RMT that was used by Nunnally *et al.*[4,6] (data not shown).

Cap. batch	Gel lot no.	Relative migration time (RMT)						
		10 kDa	20 kDa	35 kDa	50 kDa	100 kDa	150 kDa	225 kDa
1	1	1	1.17	1.34	1.48	1.77	1.94	2.12
1	2	1	1.17	1.33	1.47	1.76	1.94	2.12
2	1	1	1.17	1.33	1.48	1.77	1.94	2.12
2	2	1	1.17	1.33	1.47	1.76	1.94	2.12
	Average	N/A	1.17	1.33	1.47	1.76	1.94	2.12
	RSD%	N/A	0.06	0.18	0.19	0.22	0.21	0.15
Reference	min	N/A	1.15	1.30	1.43	1.67	1.82	1.97
Reference	max	N/A	1.17	1.33	1.47	1.75	1.92	2.10
Cap. batch	**Gel lot no.**	**RMT RSD%**						
		10 kDa	20 kDa	35 kDa	50 kDa	100 kDa	150 kDa	225 kDa
1	1	N/A	0.04	0.05	0.06	0.06	0.06	0.04
1	2	N/A	0.06	0.09	0.10	0.11	0.10	0.09
2	1	N/A	0.04	0.08	0.09	0.12	0.10	0.10
2	2	N/A	0.03	0.06	0.07	0.08	0.07	0.07
Reference	min	N/A	0.02	0.04	0.03	0.03	0.04	0.04

Table 1
Relative migration times (RMT) of molecular weight markers with the SDS Gel Buffer from Beckman Coulter. Shown are averages and repeatabilities (n = 6) measured with two capillary batches and two gel lots each. RMT values were calculated relative to the 10 kDa Internal Standard. Reference data were taken from Nunnally et al.[4] (in grey). These reference data are averages and repeatability's (n = 6) obtained in a cross-organization collaboration exercise among eight independent organizations. Shown are the data for the organization that obtained the minimal (min) and maximal (max) value, respectively.

Cap. batch	Gel lot no.	Relative migration time (RMT)						
		10 kDa	20 kDa	35 kDa	50 kDa	100 kDa	150 kDa	225 kDa
1	1	1	1.14	1.26	1.37	1.57	1.70	1.82
1	2	1	1.13	1.26	1.36	1.56	1.70	1.82
2	1	1	1.13	1.25	1.36	1.56	1.69	1.81
2	2	1	1.13	1.25	1.35	1.55	1.69	1.80
	Average	N/A	1.13	1.26	1.36	1.56	1.70	1.81
	RSD%	N/A	0.28	0.38	0.44	0.44	0.46	0.52
Cap. batch	**Gel lot no.**	**RMT RSD%**						
		10 kDa	20 kDa	35 kDa	50 kDa	100 kDa	150 kDa	225 kDa
1	1	N/A	0.03	0.06	0.06	0.08	0.11	0.09
1	2	N/A	0.01	0.02	0.02	0.02	0.03	0.03
2	1	N/A	0.02	0.04	0.03	0.03	0.03	0.05

Table 2
Relative migration times of molecular weight markers with the Protigel P from Advanced Analytical. Shown are averages and repeatability's (n = 6) measured with two capillary batches and two gel lots each.

The BSA molecular weight obtained with the gel from Beckman Coulter was between 65.5–65.7 kDa and for the Protigel P from Advanced Analytical between 70.1–72.0 kDa (Table 3). In both cases, the target of 66.4 kDa[8] was matched within an error of ± 10%.

Similarly to the RMT repeatability, the BSA molecular weight determination repeatability with the Protigel P (0.12–0.27 RSD%) was better compared to the Beckman Coulter gel (0.31–0.74 RSD%; Table 3). On multi-capillary instruments molecular weight determination repeatabilities between 1.5–2 RSD% were reported for different proteins[5]. Very similar results were obtained for both gel types with the different capillary batches and gel lots employed. The RMT precision across the four capillary batch/gel lot combinations tested were in all cases better than 0.6 RSD% (Tables 1–3).

Quantification of main IgG components

The main components of an IgG standard were quantified under reducing conditions with the CGE gels from Beckman Coulter and Advanced Analytical, respectively. Again, each set of analyses was done with two capillary batches and two gel lots. Example electropherograms are shown in Figure 4 and results are summarized in Tables 4 and 5.

The IgG standard employed contained three main components: light chain (LC), non-glycosylated heavy chain (NG) and heavy chain (HC). Peaks corresponding to these components could be identified in electropherograms obtained with the gels from both suppliers (Figure 4). However, these electropherograms showed some obvious differences.

First, the peak height ratio of HC/LC differs between the two analyses. Consistent with the results of Nunnally *et al.*[4], similar peak heights

Cap. batch	Gel lot no.	SDS Gel Buffer (Beckman Coulter)				
		RMT BSA	RMT RSD%	R-squared	MW BSA (kDa)	MW RSD%
1	1	1.59	0.13	0.9999	65.5	0.50
1	2	1.59	0.08	0.9999	65.7	0.31
2	1	1.59	0.19	0.9999	65.7	0.74
2	2	1.59	0.14	0.9999	65.6	0.56
	Average	1.59	N/A	0.9999	65.6	N/A
	RSD%	0.23	N/A	< 0.01	0.11	N/A
Reference	min	1.50	0.07	0.995	70.2	0.28
Reference	max	1.57	0.58	0.996	72.8	4.02
Cap. batch	**Gel lot no.**	**Protigel P (Advanced Analytical)**				
		RMT BSA	RMT RSD%	R-squared	MW BSA (kDa)	MW RSD%
1	1	1.47	0.03	0.9991	70.1	0.16
1	2	1.47	0.05	0.9991	71.1	0.27
2	1	1.46	0.03	0.9990	72.0	0.14
2	2	1.46	0.02	0.9989	70.7	0.12
	Average	1.47	N/A	0.9990	71.0	N/A

Table 3
Molecular weight determination of BSA by CGE with gels from Beckman Coulter and Advanced Analytical. Shown are averages and repeatability's (n = 6) measured with two capillary batches and two gel lots each. R-squared values reflect the quality of the fit of the data to a 3rd order polynomial trendline in a plot of protein marker molecular weight against average RMT (cf. Fig. 3 and Tables 1 and 2). The molecular weight of BSA was calculated from this trendline by using the RMT of BSA. Reference data were taken from Nunnally et al.[4] (in grey; cf. Table 1).

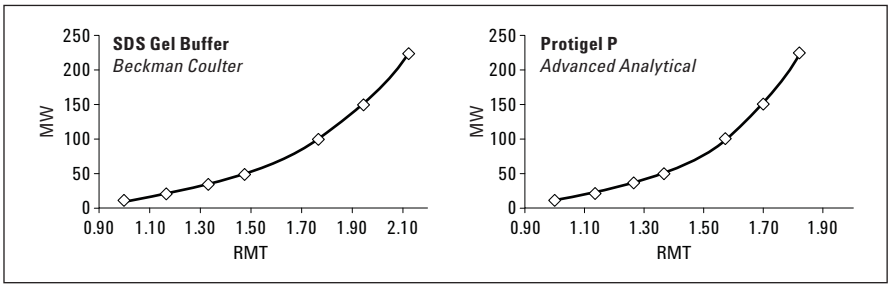

Figure 3
Representative plots for the molecular weight determination of BSA. Data were fitted to a 3rd order polynomial trendline.

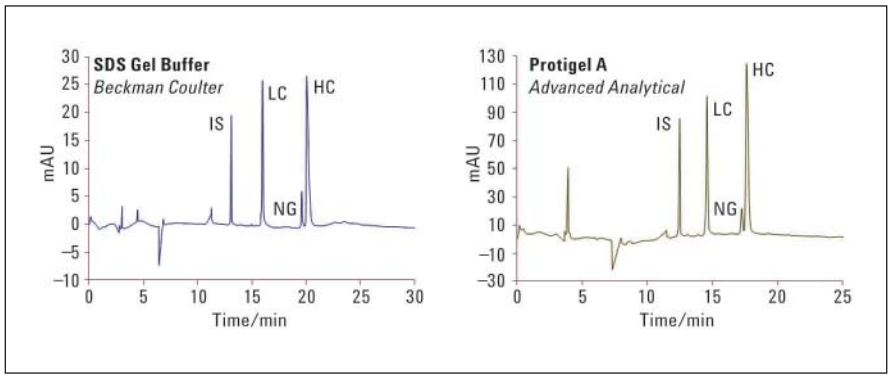

Figure 4
Representative electropherograms of the IgG sample under reducing conditions. IS, internal standard; LC, light chain; NG, non-glycosylated heavy chain; HC, heavy chain.

of LC and HC were observed with the gel from Beckman Coulter. The HC/LC peak height ratio with the Protigel A from Advanced Analytical was about 1.2 and, hence, closer to the *ideal* value of 2 that could be expected due to the IgG structure. A reason for this difference could be an increased HC peak broadening seen with the gel from Beckman Coulter, for example, due interaction with the capillary wall. An improved HC/LC ratio was reported for the Optima Protein Run Buffer from Groton Biosystems as well[6].

Additionally, with the Protigel A the NG and HC peaks were clearly separated but not baseline-resolved as with the Beckman Coulter gel (cf. Tables 4 and 5). In line with the results of Nunnally *et al.*[6], no or a much lower resolution of these peaks was obtained with the Optima Protein Run Buffer from Groton Biosystems (data not shown). Therefore, this separation matrix was not included into this study.

Results obtained with the Beckman Coulter gel were in terms of RMT very close to, and in terms of RMT repeatability with 0.02 – 0.07 RSD% in the range of the data reported by Nunnally *et al.*[4] (Table 4). However, the relative peak areas were different: peak areas of LC, NG and HC observed by Nunnally *et al.*[4] were around 30%, 9% and 62%; the respective values determined in this work with the Beckman Coulter gel were 35%, 6.5% and 58%. This discrepancy could be due to the different IgG standard lots used in both studies. For the IgG standard lot used in this work a reference value of 9.6% of relative peak area of NG compared to total heavy chain (NG+HC) was specified in the certificate of

Cap. batch	Gel lot no.	RMT			Peak areas (%)			Resolution
		LC	NG	HC	LC	NG	HC	NG/HC
1	1	1.22	1.50	1.53	35.1	6.3	58.6	1.7
1	2	1.22	1.50	1.53	35.5	6.8	57.7	1.7
2	1	1.22	1.50	1.53	34.9	6.3	58.8	1.7
2	2	1.22	1.49	1.53	35.2	6.5	58.3	1.7
	Average	1.22	1.50	1.53	35.2	6.5	58.4	1.7
	RSD%	0.09	0.11	0.14	0.63	3.9	0.79	2.3
Reference	min	1.19	1.44	1.47	27	7	58	N/A
Reference	max	1.20	1.49	1.52	35	9	64	N/A
Cap. batch	**Gel lot no.**	**RMT RSD%**			**Peak area RSD%**			**Res. RSD%**
		LC	NG	HC	LC	NG	HC	NG/HC
1	1	0.02	0.03	0.05	1.2	0.9	0.6	1.2
1	2	0.04	0.05	0.07	1.2	0.6	0.7	1.8
2	1	0.03	0.05	0.05	2.0	1.3	1.1	1.1
2	2	0.03	0.05	0.07	1.3	0.5	0.7	1.6
Reference	min	0.03	0.05	0.05	0.4	0.7	0.3	N/A

Table 4
Quantification of main antibody components under reducing conditions with the SDS Gel Buffer from Beckman Coulter. Shown are averages and repeatability's (n = 6) measured with two capillary batches and two gel lots each. Reference data were taken from Nunnally et al.[4] (in grey; cf. Table 1).

Cap. batch	Gel lot no.	RMT			Peak areas (%)			Resolution
		LC	NG	HC	LC	NG	HC	NG/HC
1	1	1.17	1.38	1.41	37.2	6.4	56.4	1.4
1	2	1.17	1.38	1.41	36.4	6.2	57.4	1.4
2	1	1.17	1.38	1.41	37.2	6.1	56.7	1.3
2	2	1.17	1.39	1.42	37.9	6.0	56.1	1.4
	Average	1.17	1.38	1.41	37.2	6.2	56.6	1.4
	RSD%	0.22	0.46	0.51	1.6	2.5	0.95	2.9
Cap. batch	**Gel lot no.**	**RMT RSD%**			**Peak area RSD%**			**Res. RSD%**
		LC	NG	HC	LC	NG	HC	NG/HC
1	1	0.10	0.21	0.25	2.0	1.6	1.2	3.7
1	2	0.08	0.17	0.19	1.4	0.5	0.9	3.9
2	1	0.14	0.30	0.33	1.7	0.6	1.1	3.1

Table 5
Quantification of main antibody components under reducing conditions with the Protigel A from Advanced Analytical. Shown are averages and repeatability's (n = 6) measured with two capillary batches and two gel lots each.

analysis of the manufacturer. This value was matched reasonably well (6.5 / { 58 + 6.5 } × 100 = 10.1%).

Another reason for the discrepancy in IgG compound quantification could be the use of different integration algorithms used by Nunnally *et al.*[4] and in this work. The peak area repeatability with the Beckman Coulter gel was with 0.6–2.0 RSD% in the range of the data reported by Nunnally *et al.*[4] (Table 4).

With the Protigel A from Advanced Analytical the RMT values were different in comparison to the Beckman Coulter gel for the same reasons as already discussed for the Protigel P (see above). In contrast to the Protigel P, the RMT repeatability with the Protigel A was between 0.08 – 0.47 RSD% and hence significantly worse compared to the data obtained with the Beckman Coulter gel (Tables 4 and 5). This lower repeatability was due to a systematic drift to increased RMT values within one sequence of runs using the same inlet and outlet buffer vials (data not shown). One reason for this drift could be buffer depletion. Better repeatabilities could possibly be obtained by changing inlet and outlet buffer vials more frequently than every eight runs or by switching of inlet and outlet buffer vial after every run.

Relative peak areas for the main IgG components observed with the Protigel A were slightly different from the respective values obtained with the Beckman Coulter gel (Tables 4 and 5). The main reason for this discrepancy was the different detection wavelength used in both cases (data not shown; cf. Experimental).

Peak area repeatabilities with the Protigel A were, on the other hand, very similar compared to the Beckman Coulter gel (Tables 4 and 5). Therefore it can be concluded that the lower resolution of NG and HC obtained with the Protigel A did not interfere with the reliable integration and quantification of these peaks. These measurements showed only a marginal dependence on the capillary batch or gel lot used. Across all capillary batch/gel lot combinations tested the RMT precision was better than 0.6 RSD% and the peak area precision better than 4 RSD% (Tables 4 and 5).

Low level impurity detection

A low level contamination was simulated by spiking low amounts of β-lactoglobulin (bLG) into a non-reduced IgG standard sample. Samples containing 0%, 0.1% and 1% bLG were analyzed with the CGE gels from Beckman Coulter and the Protigel A from Advanced Analytical, respectively. The detection of the 0.1% spike was possible with the gels from both suppliers (Figure 5).

In both cases, this spike concentration was close to the limit of detection. Given a total sample protein concentration of about 1 mg/mL, a 0.1% spike corresponds to a concentration of 1 µg/mL.

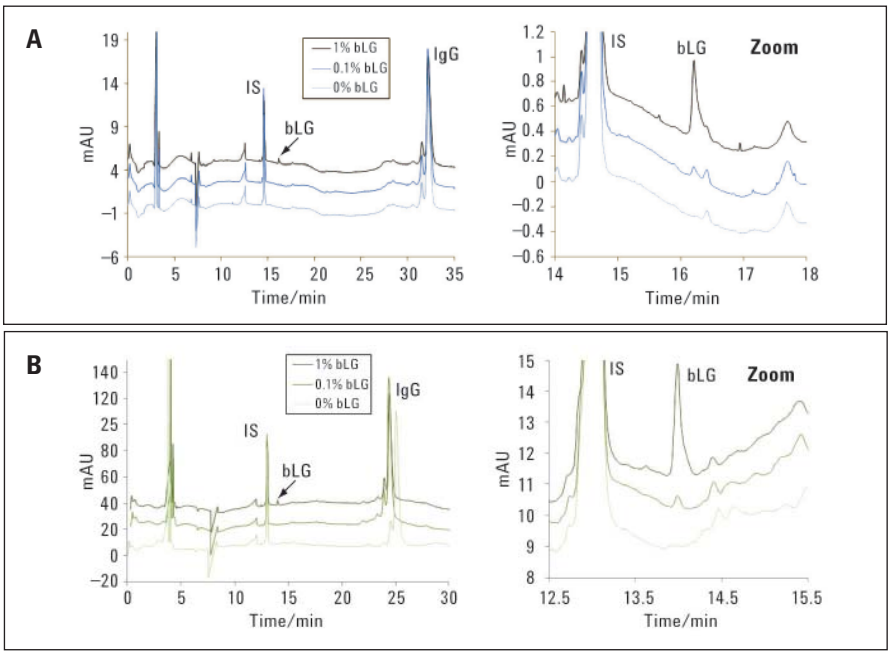

Figure 5

Low level impurity detection with the IgG sample with spiked-in bLG under non-reducing conditions. Representative electropherograms with a zoom on bLG are shown for IgG samples containing 0 %, 0.1 % and 1 % bLG, respectively, that were obtained (A) with the SDS Gel Buffer from Beckman Coulter and (B) with the Protigel A from Advanced Analytical. The time axes of the electropherograms were aligned with respect to the internal standard. IS, internal standard; bLG, β-lactoglobulin.

Conclusion

Just as the previous model, the Agilent 1600 CE system[3], the Agilent 7100 CE system is well suited for protein characterization by CGE with UV detection using commercially available separation matrices. Gels from two different suppliers tested on this system showed a similar performance in typical CGE applications like molecular weight determination, IgG component quantification and impurity detection. Consistent results were obtained across all capillary batches and gel buffer lots tested. With the gels from both suppliers, it was possible to detect a low molecular weight protein that was spiked into an intact IgG sample down to a level of 0.1%.

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